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Methods for the determination of partition coefficients based on the effect of solutes upon membrane structure

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General procedures are presented for the determination of membrane-water partition coefficients (K_p) based on the measurement of effects that depend on the extent of partitioning. Two cases are dealt with: (1) the general case when the effect is an unknown function of the total number of moles of partitioning solute (n_T) and (2) the particular case when the effect is a linear function of n_T. The procedure involves the evaluation of the effect as a function of n_T for a variety of membrane volumes (V_M), at constant volume of aqueous phase (V_{H_2O}), generating a family of curves. It is shown that when a given set of n_T, V_M produces the same effect, which indicates that the concentration of the solute in the membrane (n_M/V_M) is the same for the whole set, a plot of n_T versus V_M yields a straight line that allows the calculation of K_p. In the particular case when the effect is linearly proportional to n_T, the higher V_M, the smaller the slope. In this case, these slopes can be used to calculate K_p by plotting their inverse as a function of V_M. These procedures were applied to the partitioning of a series of compounds, some of them with known K_p, in egg phosphatidylcholine (EPC) membranes. The interaction of those compounds with the membrane was monitored either by fluorescence spectroscopy (by measuring either the excimer/monomer ratio or the quenching of intensity of an intercalated probe) or by ESR making use of a lipid spin probe. Compounds with known K_p yielded values in good agreement with previously found ones. In principle, the procedures are applicable whenever it is possible to measure a property that depends on the extent of partitioning, allowing the determination of K_p from raw data. In particular, it is very adequate for the use of spectroscopic techniques, one main advantage being that no separation of membrane and aqueous phases is required.

Introduction

In early membrane work not much attention was paid to the fact that solutes partition between the membrane and the aqueous phase and it is not uncommon to find studies where effects were analyzed without taking into account the actual amount of solute in the membrane.

More recently, this issue became much more fully acknowledged. However, the determination of partition coefficients, especially in the system under study, is still not a very common practice. In many cases, authors resort to *n*-octanol-water K_p values or to a simple relationship between the latter and those for a membrane. This approach, however, is not always valid [1].

Partition coefficients have been frequently measured by centrifugation and determination of solute concentration in the aqueous phase. This procedure can lead to errors due to the possibility of some membrane remaining in the supernatant, as well as solute co-sedimentation without true partitioning [2]. Moreover, if some chemical reaction takes place during the time of centrifugation [3,4], the method becomes inapplicable.

In fact, methods based on the separation of membrane and aqueous phases have raised a lot of controversy [5,6].

Some procedures have been presented for the determination of K_p without requiring phase separation [7–22], most of them based on fluorescence measurements [7–20]. Equations have been derived for NMR data when the exchange of the partitioning species

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between the membrane and the aqueous phase is fast in the time scale of the experiment; one method involves variations of the aqueous phase volume [21], while the other involves variation of the membranous phase volume [22].

In this report, we present general formalisms that can be applied to any kind of measurement providing information about concentration-dependent effects of partitioning solutes upon either membrane properties or those of a totally incorporated probe. Two cases are analyzed: (1) the effect is an unknown function of the total number of moles (n_T) of solute added; (2) the particular case when the effect is a linear function of n_T.

Materials and Methods

Egg phosphatidylcholine (EPC) was extracted and purified according to Ref. 23, as modified by Kamp et al. [24]. The fluorescent probe, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (10-PyPC) was obtained from Molecular Probes, Eugene, OR. Tetracaine (TTC), procaine (PRC) and 5-doxyl methyl stearate (5-MeSL) came from Sigma Chemical Co., St. Louis, MO. Tetracaine analogs containing H-(H-TTC), Cl- (Cl-TTC) and CH_3-(CH_3-TTC) substituents at the para positions were synthesized according to Ref. 25. *N*-Methylbenzhydrylamine (MBA) was synthesized according to Ref. 26 and shown to have local anesthetic activity by Consolini et al. [27]. All other compounds were reagent grade.

Lipid multibilayers containing either 10-PyPC (2.3 or 3.3 mol%) of 5-MeSL (1 mol%) were prepared by evaporating stock chloroform solutions of EPC and probe under a stream of wet nitrogen. The samples were left under vacuum for no less than 2 h. Multilamellar liposomes were obtained upon addition of 0.12 M carbonate-bicarbonate buffer (pH 10.5) prepared with doubly distilled deionized water. Phospholipid concentration was determined according to Ref. [28].

Fluorescence spectra were obtained with a Perkin Elmer LS-5 spectrofluorimeter at room temperature (23 ± 2° C). The excitation wavelength was 337 nm and the monomer and excimer emission wavelengths were 376 and 474 nm, respectively.

ESR spectra were obtained either in a Varian E-4 or in a Bruker ER-200 spectrometer at room temperature (23 ± 2° C). Lipid dispersions were placed in flat quartz cells for aqueous solution from James Scanlon, Costa Mesa, CA.

Results

The partition coefficient of a solute S is defined as

$$K_p = \frac{n_M/V_M}{n_{H_2O}/V_{H_2O}} \quad (1)$$

where n is the number of moles of solute and V the volume. The subscripts $_M$ and $_{H_2O}$ indicate membrane and aqueous phase, respectively, and $n_M + n_{H_2O} = n_T$. K_p can be calculated from measurements of any property, either of the membrane or of a totally incorporated probe, that will depend upon the extent of incorporation of the solute, S. We will refer to the measured 'effect'.

Case I: The relationship between the 'effect' and the intramembrane solute concentration is unknown

Let us assume that the magnitude of the effect observed is only determined by the intramembrane concentration of S. When measurements are performed at variable n_T, the magnitude of the effect will depend upon the values of V_m (Fig. 1a). A constant effect (dashed lines in Fig. 1a) indicates that the concentration of the solute in the membrane ($n_M/V_M = C_M$) is the same for all curves. This generates a set of n_T and V_M values such that the numerator in Eqn. 1 is constant

$$K_P = \frac{C_M}{(n_T - n_M)/V_{H_2O}} \quad (2)$$

It is also clear that the aqueous concentration of S is the same for the whole n_T, V_M set. Rearranging Eqn. 2 yields

$$n_T = \frac{C_M \cdot V_{H_2O}}{K_p} + C_M V_M \quad (3)$$

Thus a plot n_T vs. V_M yields a straight line (Fig. 1b) that allows the evaluation of the membrane solute concentration that produces the effect under consideration, and the value of K_p from

$$K_p = \frac{\text{slope} \cdot V_{H_2O}}{\text{intercept}} \quad (4)$$

This procedure provides values of K_p at any selected value of the effect (and hence at any value of C_M) The method can be applied both to partition constants showing 'non-ideal' behavior as well as to processes showing saturation of solubilization sites (i.e., to the evaluation of 'binding' constants [18]).

Case II: The 'effect' is linearly proportional to n_T

Equation 1 can be rearranged to show that an effect that is linearly proportional to the solute concentration in the membrane (n_M/V_M) will yield a straight line in a plot of 'effect' versus n_T. The effect is then given by $\beta \cdot (n_M/V_M)$, where β is the proportionality constant.

The plot of 'effect' versus n_T at constant V_m and V_{H_2O} is described by Eqn. 5:

$$\text{'effect'} = \beta \cdot \left(\frac{n_M}{V_M}\right) = \beta \cdot \frac{K_p}{V_{H_2O} + K_p V_M} \cdot n_T \quad (5)$$

The lower the membrane concentration, the greater the slope, α, where

$$\alpha = \frac{\beta \cdot K_p}{V_{H_2O} + K_p V_M} \quad (6)$$

If experiments are performed for a series of membrane volumes, a family of straight lines will be obtained whose slopes (α) will decrease as V_M increases as illustrated in Fig. 3a. Equation 6 can be converted into

$$\frac{1}{\alpha} = \frac{V_{H_2O}}{\beta \times K_p} + \frac{V_M}{\beta} \quad (7)$$

which shows that a plot of $1/\alpha$ versus V_M yields a straight line (Fig. 3b) and that K_p can be obtained from the slope and intercept of such a plot, as in case 1 (Eqn. 4).

This analysis was found to apply to the partitioning of a series of compounds into EPC multilamellar liposomes (Table I) as monitored by fluorescence and ESR spectroscopy.

The fluorescence measurements made use of the excimer/monomer ratio of intensities of a pyrene-containing phospholipid (10-PyPC) to evaluate the partitioning of *n*-alkanols into EPC multilamellar liposomes and of the quenching of probe fluorescence to determine the K_p of the local anesthetic tetracaine (TTC). For ESR, the spectra of an intercalated spin probe, 5-MeSl, were used to analyze the partitioning of a series of compounds with local anesthetic activity.

Fig. 1 shows the effect of *n*-nonanol addition upon the relationship between the excimer (measured at 474 nm) and monomer (measured at 376 nm) fluorescence intensities of 10-PyPC. Since the probe is totally incorporated into to the liposomes, the values of I_{474}/I_{376} measured in the absence of *n*-nonanol are independent of membrane concentration. These values increase in the presence of *n*-nonanol in a membrane concentration-dependent fashion. Values of $(I_{474}/I_{376})/(I_{474}/I_{376})_o$ for *n*-octanol and *n*-decanol show a pattern similar to that of Fig. 1a. The increase in excimer emission can be related to a decrease in viscosity, rendering the diffusion-controlled excimer formation more favourable. If it is assumed that the effect observed is only determined by the intravesicular *n*-alkanol concentration, the value of K_p can be calculated according to the formalism presented.

The values of K_p obtained for the three *n*-alkanols studied are given in Table I.

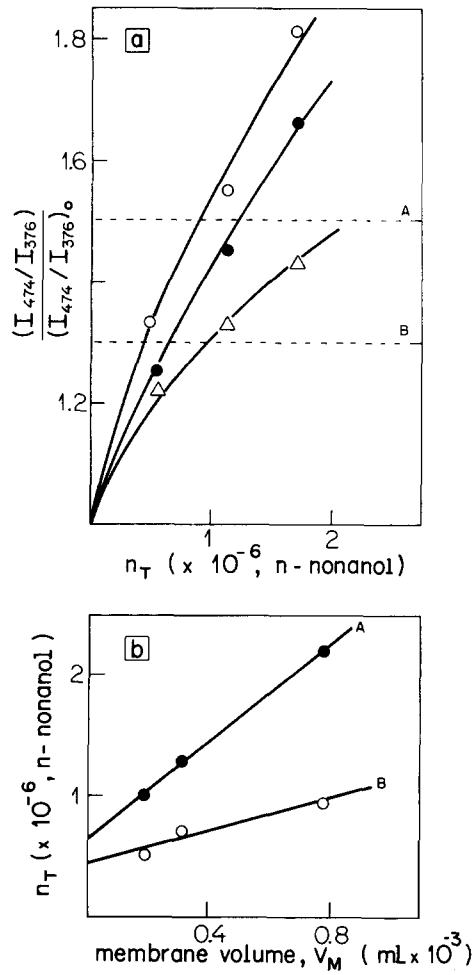

Fig. 1. (a) Effect of 1-nonanol on the excimer/monomer fluorescence intensity ratio (I_{474}/I_{376}) of 10-PyPC at variable EPC concentration (mg/ml): (○) 0.20, (●) 0.31, (△) 0.78. $(I_{474}/I_{376})_o$ is obtained in the absence of *n*-nonanol. The dashed lines parallel to the abscissa (A and B) generate two sets of n_T, V_M values for which the effect is the same. (B) Plot of n_T vs. V_M for the sets generated by the dashed lines in (a) (A and B). $V_{H2O} = 1.0$ ml, V_M is obtained as described in the text.

It is seen that by this procedure values of the partition constant for the *n*-alkanols can be obtained independently of the mechanism by which they modify the probe fluorescence spectra, and independently of the relationship between the observed effect and the *n*-alkanol concentration. Nevertheless, it can be seen that, due to the large amounts of alkanols needed to elicit a significant effect, the partition coefficient is obtained when large amounts of alkanols are incorporated. On the other hand, when the solute is able to quench the probe fluorescence, the partition can be determined at considerably lower intramembrane solute concentrations. The data given in Fig. 2 show the results obtained by measuring with the same probe, the effect of TTC upon the monomer fluorescence intensity. In this figure, values of $(I_{376})_o/(I_{376})$ are plotted as a function of TTC concentration at several membrane concentrations. K_p obtained by this procedure is also given in Table I.

TABLE I

Partition coefficients (K_p) of a series of compounds in EPC multilamellar liposomes determined according to the procedures described in the text

Compound	Method		
	fluorescence	ESR	other
n-Octanol	800		700 [c]
n-Nonanol	3000 [a]		
	2900 [b]		
n-Decanol	6000		
TTC	1100	780	710 [d]
Cl-TTC		610	
H-TTC		420	
CH_3-TTC		580	
PRC		95	59 [d]
MBA		120	

[a] Obtained from curve A in Fig. 1b.
[b] Obtained from curve B in Fig. 1b.
[c] Octanol-water partition coefficient, Ref. 29.
[d] Obtained by spectrophotometric measurement of the solute concentration in the aqueous phase after centrifugation (from Ref. 22, corrected according to Ref. 30).

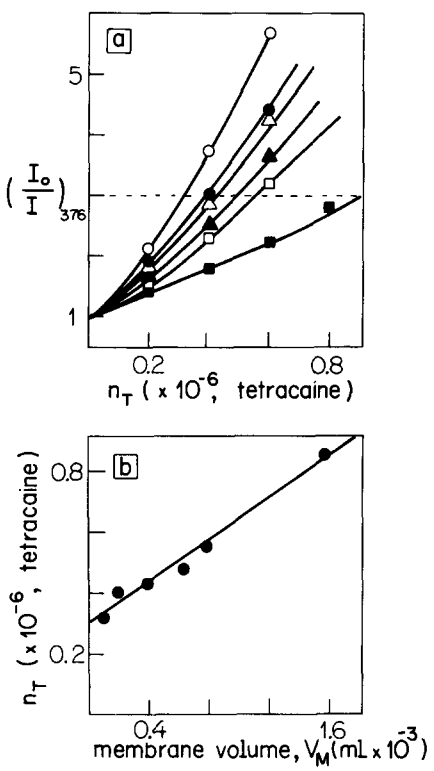

Fig. 2. (a) Effect of TTC upon the fluorescence intensity (I) of 10-PyPC at variable EPC concentration (mg/ml): (○) 0.10, (●) 0.20, (△) 0.39, (▲) 0.63, (□) 0.78, (■) 1.56. I_o is obtained in the absence of TTC. The dashed line parallel to the abscissa generates a set of n_T, V_M values for which the effect is the same. (b) Plot of n_T vs. V_M for the set generated by the dashed line in (a). $V_{H_2O}=1.0$ ml, V_M is obtained as described in the text.

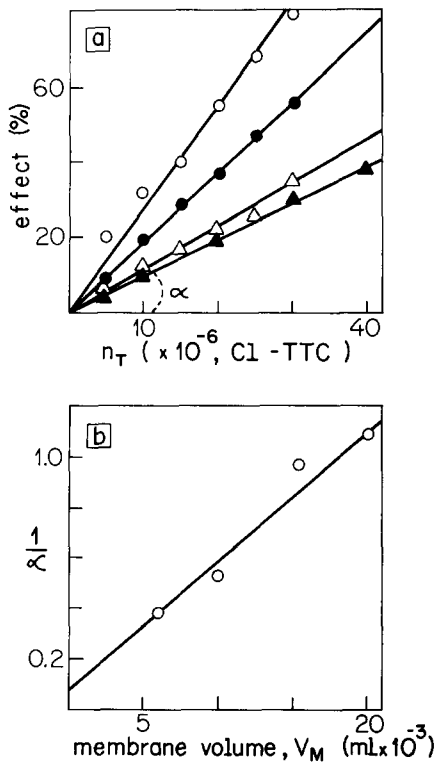

Fig. 3. (a) Effect of Cl-TTC upon the ESR spectra of 5-MeSL at variable EPC concentration (mg/ml): (○) 6.0, (●) 10.0, (△) 15.4, (▲) 20.0. The ordinates indicate the percent change in $(h_{+1}/h_o)_o$, the ratio of heights of the low-field to the center-field resonances in the absence of Cl-TTC. (b) Plot of the reciprocal of the slopes (α) of the lines in (a) as a function of membrane volume $V_M=1.0$ ml, V_M is obtained as described in the text.

Analysis of the ESR data made use of the fact that partitioning led to changes in membrane organization which in turn, led to changes in the spectra of 5-MeSL. Membrane organization was assessed by the parameter h_{+1}/h_0, the ratio of the low field to center field resonances. It has been previously discussed that this parameter contains the contribution of both order and mobility and is taken as expressing changes in overall organization [30,31]. Fig. 3 (a and b) illustrates the use of Eqns. 5 and 7, for the analysis of the partitioning of Cl-TTC. Upon addition of Cl-TTC, h_{+1}/h_0 increases, indicating a decrease of membrane organization (Fig. 3a). Fig. 3b shows the plot of the reciprocal of the slopes of the lines in Fig. 3a versus membrane volume. The membrane volumes in Figs. 1b–3b were calculated by taking the lipid density equal to 1 g/ml [32]. Similar procedures were applied to TTC, its other analogs, PRC and MBA. The calculated values of K_p are given in Table I. A comparison between the present results and data in the literature for TTC and PRC reveals good agreement.

Discussion

The formalisms presented here are (necessarily) similar to previously published ones [7–22]. Most of the previous work was based on the measurement of fluorescence quenching and most of the expressions for calculation of K_p required the knowledge of the absolute value of some parameter.

The main contribution of the present derivations is that the analysis requires only raw data, as long as the data report on the extent of a given effect (proportional to the concentration of the solute in the membrane) as a function of the total number of moles of the partitioning species. This means that any property, be it of the membrane or of a totally incorporated probe, that is altered as a function of partitioning, can be used to determine K_p.

Experiments are performed by analyzing an effect as a function of the total concentration of solute added, for a given membrane concentration, and then similar experiments are done for different membrane concentrations.

The formalisms presented are clearly very suitable for the use of spectroscopic techniques.

Figs. 1 and 2 illustrate the use of fluorescence measurements of a fluorescent phospholipid probe to determine K_p of an alkanol (Fig. 1) and of a local anesthetic (Fig. 2). In both cases the measured property (excimer/monomer intensity ratio, Fig. 1, and intensity quenching, Fig. 2) did not vary linearly as a function of solute total concentration. Nevertheless, the formalism presented in Case I (Results) enables the analysis of the data to derive K_p.

The ESR measurements of the degree of organization of the lipid spin probe 5-MeSL yielded straight lines when the effect of compounds with local anesthetic activity was examined (Fig. 3). In this case, the data could be analyzed according to the formalism presented in Case II (Results). Case II is obviously a particular case of the more general case, I, the underlying events being the same in both cases.

An important point is that the above methods enable the determination of K_p for any solute in the membrane under study without requiring phase separation.

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